## REMARKS

Claims 43-108 are pending in the present application.

Applicants would like to thank Examiner Baum and Examiner Bui for the extremely helpful and productive discussions with their undersigned Representative on September 11, 12, and 17, 2003 regarding U.S. Application Serial No. 09/824,734. Applicants would also like to thank the Examiners for assisting in the preparation of the claim language reflected hereinabove, which the Examiners have indicated are free from the criticisms under 35 U.S.C. §112, first paragraph, in this application. For the same reasons that these claims are compliant with 35 U.S.C. §112, first paragraph, in that application so too are the claims compliant in the present application. The content of these discussions are reflected in the comments and amendments set forth herein.

Applicants would like to thank Examiner Baum for the indication that SEQ ID NO: 1 and 2 are free from the art of record. In addition, Applicants wish to thank Examiner Baum for withdrawing the rejection under 35 U.S.C. §112, second paragraph. Reconsideration is respectfully requested in view of the following comments and the amendments presented herein.

The rejections of Claims 5-8 under 35 U.S.C. § 112, first paragraph ("written description"), and of Claims 1-22 and 32-35 under 35 U.S.C. §112, first paragraph ("enablement") are obviated by amendment.

The Office has alleged that the specification fails to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of

the claimed invention (paper number 10, page 8, lines 3-5). It appears that this ground of rejection is based on the breadth of the claims that were initially presented coupled with the absence of a "description of domains that are specific to this particular serine/threonine kinase nor domains that are important for its proper function."

Applicants have amended the claims to avoid such criticism and to narrow the scope of homology to embrace the polynucleotide sequence of SEQ ID NO:1, polynucleotide sequences encoding SEQ ID NO:2 and polynucleotide sequences encoding proteins having 95% homology to SEQ ID NO:2 where the homologs serine/threonine kinase activity.

The serine/threonine kinase activity and methods of screening for this activity is exemplified in the specification at page 12, line 18 to page 16, line 26. Applicants submit that the specification provides an adequate description to allow the skilled artisan to recognize what has been invented and what is claimed is adequately described in the specification within the meaning of 35 U.S.C. § 112, first paragraph (see MPEP § 2163.02

Moreover, Applicants submit that determining what sequences fall within or without the scope of the present claims would be readily apparent to the skilled artisan. At page 12, line 18 to page 16, line 26, Applicants provide a detailed example of how the skilled artisan may clone, express, and characterize any sequence variant to assess its standing with respect to the claimed invention. Therefore, with the present specification in hand the artisan may readily practice the present invention without undue experimentation (see MPEP § 2164.01).

Based on the foregoing, Applicants submit that the present claims are fully described and enabled by the specification and the common knowledge available in the art and as such withdrawal of these grounds of rejection is requested.

The rejection of Claims 1-22 and 32-35 under 35 U.S.C. § 101 is traversed.

Applicants submit that the present invention provides, in part, nucleotide sequences for the sos2 gene, amino acid sequence for the SOS2 protein, methods of making the SOS2 protein, methods of making a transgenic plant comprising introducing the polynucleotide encoding the sos2 gene, methods of screening for polynucleotides which encode a protein having serine/threonine kinase activity, and a method of increasing salt tolerance in a plant in need thereof (pages 4-5).

Applicants once again note that the Examiner has improperly applied the "credible utility" analysis. MPEP §2107.02 states:

> An applicant need only make one credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. 101 and 35 U.S.C. 112; additional statements of utility, even if not "credible," do not render the claimed invention lacking in utility. See, e.g., *Raytheon v. Roper*, 724 F.2d 951, 958, 220 USPQ 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984).

When this standard is applied, Applicants submit that the present application is in full compliance with the 35 U.S.C. §101. Applicants note that the "need only make one credible assertion" test is met by each of the objects highlighted above.

Citing *In re Gaubert*, the Examiner appears to be requiring additional evidence to support the asserted ultimate utility of the present invention of increasing salt tolerance by expressing the SOS2 gene in a plant in need thereof. More precisely, the Examiner appears to be interjecting his own opinion on the state of the art in which he asserts that the "art teaches the involvement of protein kinases in a wide array of biological process, except in relation to salt tolerance."

Applicants submit that the Examiner has clearly deviated from the procedure necessary to shift the burden of proof on the Applicant and, as such, Applicants need not provide any further evidence to support its asserted utility. The procedure that the Examiner

must satisfy appears in MPEP §2107.02, which states:

> An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. ...
>
> To properly reject a claimed invention under 35 U.S.C. 101, the Office must (A) make a *prima facie* showing that the claimed invention lacks utility, and (B) provide a sufficient evidentiary basis for factual assumptions relied upon in establishing the *prima facie* showing. *In re Gaubert*, 524 F.2d 1222, 1224, 187 USPQ 664, 666 (CCPA 1975)

Applicants submit that the Examiner has, in no way, provided even a shred of evidence to support a prima facie showing that the claimed invention lacks utility. In particular, the Examiner has failed to provide a "sufficient evidentiary basis" or even attempt to "make a prima facie showing that the claimed invention lacks utility" for each and every asserted utility noted above.

All the Examiner has set forth in the present rejection can best be summarized with the following statement: "I don't believe you, so show me." Applicants submit that if the Examiner is incapable of providing sufficient evidentiary basis for factual assumptions relied upon in establishing the *prima facie* showing, then the Examiner should place his "beliefs" and "assertions" in an Examiner's Declaration.

In an effort to expedite prosecution, Applicants **submit herewith** a manuscript by Gou et al, which has been submitted for publication and is entitled: "Transgenic evaluation of activated mutant alleles of SOS2 reveals a critical requirement of its kinase activity and C-terminal regulatory domain for salt tolerance in Arabidopsis." Applicants note that Gou et al provides the evidence that the claimed serine/threonine kinase is involved in salt tolerance and, therefore, clearly supports the asserted utility.

In view of the foregoing coupled with the additional evidence provided by Gou et al,

Applicants request withdrawal of this ground of rejection.

The objection to the specification is obviated by amendment. The specification has been amended beginning on page 12, line 18 to remove an embedded hyperlink. Applicants note that in the Amendment and Request for Reconsideration filed on February 21, 2003, this amendment was made; however, the beginning page and line number were indicated as page 6, line 19. Also in the response filed on February 21, 2003, the paragraph beginning on page 6, line 19 was properly amended and it is this amendment of that paragraph that should control. However, for sake of clarity, the paragraph beginning on page 6, line 19 is reproduced herein above.

Withdrawal of this ground of objection is requested.

Applicants submit that the application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Eckhard H. Kuesters
Attorney of Record
Registration No. 28,870

Vincent K. Shier, Ph.D.
Registration No. 50,552

22850

(703) 413-3000
Fax No.: (703)413-2220
NFO:VKS

**Transgenic evaluation of activated mutant alleles of SOS2 reveals a critical requirement of its kinase activity and C-terminal regulatory domain for salt tolerance in Arabidopsis**

Yan Guo, Quan-Sheng Qiu, Masaru Ohta, Changqing Zhang, Francisco J. Quintero[‡], José M. Pardo[‡], Karen S. Schumaker, and Jian-Kang Zhu*

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721

[‡]Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Cientificas, Sevilla 41080, Spain

*To whom correspondence should be addressed.

Running title: Active SOS2 kinases

For correspondence: Jian-Kang Zhu

Department of Plant Sciences

University of Arizona

Tucson, Arizona 85721

Tel. (520) 626-2229

Fax (520) 621-7186

E-mail: jkzhu@ag.arizona.edu

## ABSTRACT

In Arabidopsis, the calcium-binding protein SOS3 interacts with and activates the protein kinase SOS2, which in turn activates the plasma membrane $Na^+/H^+$ antiporter SOS1 to bring about sodium ion homeostasis and salt tolerance. Constitutively active alleles of SOS2 can be constructed in vitro by changing $Thr^{168}$ to Asp in the activation loop of the kinase catalytic domain and/or by removing the autoinhibitory FISL motif from the C-terminal regulatory domain. We expressed various activated forms of SOS2 in yeast and in Arabidopsis and evaluated the salt tolerance of the transgenic organisms. Experiments in which the activated *SOS2* alleles were co-expressed with *SOS1* in yeast showed that the kinase activity of SOS2 is partially sufficient for SOS1 activation in vivo, and higher kinase activity leads to a more dramatic increase in SOS1 activation. In planta assays showed that the $Thr^{168}$-to-Asp activated mutant SOS2 partially rescued the salt hypersensitivity in *sos2* and *sos3* mutant plants. In contrast, SOS2 lacking only the FISL domain suppressed the *sos2* mutation but not *sos3*, whereas truncated forms in which the C-terminal had been removed could not restore the growth of either *sos2* or *sos3* plants. Expression of some of the activated SOS2 proteins in wild-type Arabidopsis conferred increased salt tolerance. These studies demonstrate that the protein kinase activity of SOS2 is partially sufficient for activation of SOS1 and for salt tolerance in vivo and in planta, and that the kinase activity of SOS2 is limiting for plant salt tolerance. The results also reveal an essential in planta role for the SOS2 C-terminal regulatory domain in salt tolerance.

## INTRODUCTION

Soil salinity is a serious environmental stress limiting plant productivity. Sodium ions ($Na^+$), which are abundant in saline soils, are cytotoxic in plants. $Na^+$ enters plant cells through transporters such as HKT1 (Rus et al., 2001) and non-selective cation channels (Amtmann and Sanders, 1999). To prevent $Na^+$ buildup in the cytoplasm, plant cells employ $Na^+/H^+$ antiporters at the plasma membrane and tonoplast to transport $Na^+$ into the apoplast and vacuole, respectively (Apse et al., 1999; Qiu et al., 2002). Over-expression of the Arabidopsis plasma membrane $Na^+/H^+$ antiporter SOS1 or the vacuolar $Na^+/H^+$ antiporter AtNHX1 improves salt tolerance in transgenic plants (Apse et al., 1999; Zhang et al., 2001; Zhang and Blumwald, 2001; Shi et al., 2003). Enhanced salt tolerance can also be achieved by over-expression of the vacuolar $H^+$-pyrophosphatase AVP1, which generates the driving force for $Na^+$ transport into the vacuole (Gaxiola et al., 2001).

Recently, a regulatory pathway for ion homeostasis and salt tolerance was identified in Arabidopsis (Zhu, 2000, 2002). Salt stress is known to elicit a rapid increase in free calcium concentration in the cytoplasm (Knight et al., 1997). SOS3, a myristoylated calcium-binding protein, is proposed to sense this calcium signal (Liu and Zhu, 1998; Ishitani et al., 2000). SOS3 physically interacts with the protein kinase SOS2, and activates the substrate phosphorylation activity of SOS2 in a calcium-dependent manner (Liu et al., 2000; Halter et al., 2000). The SOS3-SOS2 protein kinase complex phosphorylates SOS1 in vitro, and is required for the activation of the $Na^+/H^+$ antiport activity of SOS1 in vivo (Qiu et al., 2002; Quintero et al., 2002). Loss-of-function mutations in *SOS3* and *SOS2* cause hypersensitivity to $Na^+$ (Zhu et al., 1998).

SOS2 has a highly conserved N-terminal catalytic domain similar to that of yeast SNF1 and animal AMPK (Liu et al., 2000). Within the SOS2 protein structure, the N-terminal catalytic region interacts with the C-terminal regulatory domain (Guo et al., 2001). SOS3 interacts with the FISL motif in the C-terminal region of SOS2 (Guo et al., 2001) which also serves as an autoinhibitory domain. A constitutively active SOS2 kinase, T/DSOS2, can be engineered by a $Thr^{168}$-to-Asp change (to mimic phosphorylation by an upstream kinase) in the putative activation loop. The kinase activity of T/DSOS2 is independent of SOS3 and calcium (Guo et al., 2001). Constitutively active forms of SOS2 can also be created by removing the FISL motif (SOS2DF) or the entire C-terminal regulatory domain (SOS2/308) (Guo et al., 2001; Qiu et al., 2002). The activation loop mutation and the autoinhibitory domain deletions have a synergistic effect on the kinase activity of SOS2, and super-active SOS2 kinases, T/DSOS2/308 or T/DSOS2/DF, can be created when the two changes are combined (Guo et al., 2001; Qiu et al., 2002). We have shown that T/DSOS2/DF could activate the transport activity of SOS1 in vitro, whereas the wild-type SOS2 protein could not (Qiu et al., 2002). However, whether these active forms of SOS2 can function in vivo is not known.

In this study, we expressed various activated SOS2 proteins in yeast and Arabidopsis, with the aim of determining if the protein kinase activity of SOS2 is sufficient for activation of the SOS1 plasma membrane $Na^+/H^+$ antiporter in vivo and in planta, and to identify domains in the SOS2 protein that are important for its in planta function. We also investigated if the kinase activity of SOS2 is limiting for plant salt tolerance to evaluate the potential of using the activated SOS2 mutant alleles for improving plant salt tolerance.

## RESULTS

### Changes in the SOS2 protein produce constitutively active kinases

Based on its inability to autophorphorylate or phosphorylate a peptide substrate, SOS2 appears to be an inactive kinase. The calcium-binding protein SOS3 has been shown to interact with and activate SOS2 in vitro in the presence of calcium (Halfter et al., 2000). We have previously shown that SOS2 kinases that are active in the absence of SOS3 and calcium (constitutively active SOS2) could be produced either by exchange of $Thr^{168}$ in the activation loop to the acidic residue Asp (T/DSOS2) or by deletion of the FISL motif in the C-terminal regulatory domain of the SOS2 protein (SOS2DF, Guo et al, 2001; Qiu et al, 2002), and that a super-active SOS2 kinase could be generated by combining these two changes (T/DSOS2DF, Qiu et al., 2002). In the present study, additional changes were made to the SOS2 kinase to allow us to develop a series of SOS2 proteins for studies of SOS2 structure and function. The FISL motif and C-terminal 117 amino acids or the C-terminal 117 amino acids were removed in the GST-T/DSOS2/308 and GST-T/DSOS2/329 constructs, respectively (Figure 1A). These proteins were assayed for autophosphorylation or their ability to phosphorylate a peptide substrate and their activities compared to those of wild-type SOS2 protein, T/DSOS2 or T/DSOS2DF. T/DSOS2/308 had the strongest activities, followed by T/DSOS2DF, T/DSOS2, T/DSOS2/329, and SOS2 (Figure 1B to 1D). These kinase constructs served as the basis of the following transgenic studies in yeast and Arabidopsis.

**The protein kinase activity of SOS2 is partially sufficient for salt tolerance in vivo in a heterologous system**

Recently, the Arabidopsis SOS regulatory pathway has been reconstituted in *Saccharomyces cerevisiae* (Quintero et al., 2002), providing an in vivo system for studies of SOS2 structure-function relationships. To determine if the kinase activity of SOS2 is sufficient for activation of SOS1, wild-type and constitutively active SOS2 kinases were introduced into a yeast strain in which the endogenous yeast $Na^+$ transporters ($Na^+$ efflux proteins ENA1-4 and NHA1 and the vacuolar $Na^+/H^+$ exchanger NHX1) had been removed (Quintero et al., 2002) and the Arabidopsis *SOS1* gene was expressed. The transformed yeast strains were grown on Synthetic Complete (SC) medium containing various concentrations of NaCl and the results are shown in Figure 2. Expression of SOS1 restored partially the NaCl tolerance of yeast grown at 300 mM NaCl, and this NaCl tolerance was further enhanced by the co-expression of SOS2 with SOS1, where growth could be supported up to 450 mM NaCl. There was no further increase in salt tolerance when SOS1 was co-expressed with T/DSOS2 in place of SOS2, which mimicked the phosphorylated state of SOS2. These data suggest that SOS2 may be partially activated by an unknown protein in yeast. However, expression of T/DSOS2/308 and T/DSOS2DF strongly enhanced the ability of the yeast to grow in salt concentrations up to 600 mM NaCl, with maximal growth in the presence of T/DSOS2/308. These relative tolerances are in agreement with the in vitro activity displayed by the recombinant SOS2 polypeptides (Figure 1D) and show that the kinase activity of SOS2 is partially sufficient for activation of SOS1 and salt tolerance in vivo in yeast. On the other hand, none of the active SOS2 kinases could increase the salt tolerance of yeast to the same level achieved when *SOS1* was co-transformed with both *SOS2*

and *SOS3* (data not shown), demonstrating that the active SOS2 kinases still require SOS3 for targeting to the plasma membrane and full activation of SOS1 (Quintero et al., 2002).

**The protein kinase activity of SOS2 is partially sufficient for salt tolerance in planta**

To determine if the protein kinase activity of SOS2 is sufficient for salt tolerance in planta, wild-type and the constitutively active forms of SOS2 were expressed under the CaMV 35S promoter in the *sos2* and *sos3* mutants of Arabidopsis. Five-day-old $T_2$ transgenic plants expressing *35S::SOS2* (germinated on MS medium without salt) were transferred to plates with either MS or MS with 100 mM NaCl. Three of twelve independent $T_2$ transgenic lines in the *sos2-2* background evaluated had salt tolerance nearly restored to levels equivalent to that of wild type. In contrast, none of the twenty-four independent transgenic lines in the *sos3-1* background evaluated showed any increased salt tolerance relative to the *sos3-1* mutant.

One representative $T_3$ homozygous *35S::SOS2* line in the *sos3-1* and *sos2-2* backgrounds was evaluated for *SOS2* transcript accumulation and growth in salt (Figure 3). RNA analysis indicated that the transgenic plants accumulated high levels of *SOS2* mRNA from the transgene, since the endogenous *SOS2* expression was extremely low (Figure 3E) and could only be seen with prolonged exposure of the blot (not shown). The results show that ectopic expression of SOS2 under the CaMV 35S promoter could rescue the *sos2-2* phenotype (Figure 3B). As expected, the ectopic expression of SOS2 did not rescue the *sos3-1* salt-hypersensitive phenotype (Figure 3D), confirming that the wild-type SOS2 protein must be activated by SOS3 in vivo for function in Arabidopsis.

Expression of active T/DSOS2 kinase in *sos2-2* and *sos3-1* resulted in five of twelve $T_2$ *sos2-2* transgenic lines and four of twelve $T_2$ *sos3-1* transgenic lines in which the shoot-, but not

the root-sensitivity of the mutant phenotype was rescued. RNA analysis demonstrated that the *sos2-2* and *sos3-1* transgenic plants accumulated a high level of *T/DSOS2* transcript (Figure 4A). *SOS2* transcript and protein are in low abundance in Arabidopsis and, even when *SOS2* transcript levels were higher due to the strong CaMV 35S promoter, SOS2 protein levels were still virtually undetectable using our SOS2 antisera (data not shown). Therefore, in order to analyze the levels of T/DSOS2 protein in the transgenic plants, total proteins were extracted from mutants and transgenic plants and SOS2 protein (from both endogenous *SOS2* and *35S::T/DSOS2*) was enriched based on its binding to SOS3. The proteins were loaded onto a column containing GST-SOS3 fusion protein that was bound to glutathione-Sepharose beads. The resulting GST-SOS3-SOS2 or T/DSOS2 complexes were used for either immunoblot analysis or peptide phosphorylation assays. As shown in Figure 4B, expression of T/DSOS2 in either *sos2-2* or *sos3-1* resulted in the accumulation of T/DSOS2 protein at higher levels than the pre-existing SOS2 protein levels in the *sos2-2* and *sos3-1* mutants. Based on phosphorylation of the p3 peptide, T/DSOS2 kinase activity from both the *sos2-2* and *sos3-1* transgenic plants was approximately four times higher than in the corresponding mutants (Figure 4C). Since several PKS (SOS2-like protein kinases) proteins also interact with SOS3 (Guo et al., 2001), the kinase activities from the untransformed mutants may not represent the activity of only SOS2.

Five-day-old seedlings of wild-type, mutant and T/DSOS2 transgenic plants were transferred to either MS medium or MS medium containing 100 mM NaCl. No significant differences in plant growth were observed on MS medium (Figure 5A). When the plants were grown on medium containing 100 mM NaCl, the growth of wild-type plants was retarded but root bending was largely unaffected, while growth of *sos2-2* and *sos3-1* was severely inhibited (Figure 5B) and plants died within 2 weeks (not shown). Expression of T/DSOS2 in *sos2-2* was

able to partially rescue the shoot salt hypersensitivity but not the root salt hypersensitivity (Figure 5B). These results suggest that, in the shoot, ectopic expression of T/DSOS2 partially restored salt tolerance in the *sos2-2* background. Expression of T/DSOS2 in *sos3-1* was also able to partially rescue the shoot salt hypersensitivity but not the root salt hypersensitivity (Figure 5B and 5C), suggesting that, in the shoot, addition of the active kinase partially by-passed the requirement for SOS3.

No differences in either vegetative or reproductive growth were seen when mutant and transgenic plants grown in soil were watered with 0.05× MS nutrients in the absence of NaCl (data not shown). However, when the plants were treated with NaCl, *sos2-2*, and *sos3-1* lost vigor faster, and both vegetative and reproductive growth decreased (Figure 5D and 5E). Expression of T/DSOS2 improved the growth of the mutants under NaCl stress (Figure 5D and 5E); although it did not restore salt tolerance to wild-type levels (data not shown).

**The protein kinase activity of SOS2 may be limiting for salt tolerance in Arabidopsis**

To determine if levels of SOS2 protein are limiting in vivo and if increasing active SOS2 levels leads to improved salt tolerance in planta, *SOS2* and *T/DSOS2* were expressed in wild-type plants under the control of the CaMV 35S promoter. Of twenty-four $T_2$ *35S::SOS2* transgenic lines evaluated, all had levels of salt tolerance similar to that in untransformed wild type. The levels of *SOS2* transcript were determined in three $T_3$ homozygous *35S::SOS2* lines and strong expression was detected in all the transgenic plants (Figure 6A). The salt tolerance of two of these lines was subsequently evaluated during germination (Figure 6B and 6C) and seedling growth (Figure 6D to 6F); responses to salt at both stages were similar to those in the wild type.

The lack of enhancement of salt tolerance in plants over-expressing wild-type SOS2 indicates that SOS2 protein levels are not limiting in Arabidopsis in vivo.

Exchange of $Thr^{168}$ in the activation loop of the SOS2 protein with Asp mimics the phosphorylation of $Thr^{168}$ by an unknown upstream kinase and leads to activation of SOS2 (Guo et al., 2001). When T/DSOS2 was expressed in wild-type plants, seven of thirty-four $T_2$ transgenic lines evaluated showed increased NaCl tolerance compared to untransformed wild-type plants. Two of the seven $T_3$ homozygous *35S::T/DSOS2* lines were analyzed for *T/DSOS2* transcript and protein accumulation and salt tolerance. The transgenic plants accumulated high levels of *T/DSOS2* transcript and protein (Figure 7A and 7B). T/DSOS2 kinase activity from the transgenic plants was enhanced 4-5 times over the kinase activity levels in wild type (Figure 7C). No difference was seen when seeds from transgenic or wild-type plants were germinated on MS medium without salt (Figure 8A, left panel). However, seeds from the transgenic lines showed more rapid germination on MS medium containing 100 mM NaCl (Figure 8A, right panel) and seedling development proceeded further in salt (green cotyledons developed) in the transgenic plants. Growth of wild-type and transgenic seedlings in the absence of salt was similar (Figure 8B, upper panel). However, when seedlings were transferred to medium with NaCl, the transgenic plants showed significantly less growth inhibition, which was especially evident at 120 mM NaCl (Figure 8B, middle and lower panels).

To test the salt tolerance of the plants when grown in soil, wild-type and transgenic seeds were germinated in soil and watered with 0.05× MS nutrients. After three weeks, the plants were treated with NaCl by progressively increasing the salt concentration 50 mM every 4 days until a final concentration of 200 was reached (Shi et al., 2003). The transgenic plants showed improved vegetative and reproductive growth in soil with 200 mM NaCl when compared to

growth of wild-type plants (Figure 8C); no difference was found when plants were grown without NaCl (data not shown). The increased salt tolerance of the plants expressing the T/DSOS2 kinase suggests that levels of activated kinase may be limiting in Arabidopsis in vivo and that increasing active SOS2 levels in planta can lead to improved salt tolerance.

**Enhancement of SOS1 activity in vivo by constitutively active SOS2**

Previous studies have shown that active SOS2 protein stimulates the $Na^+/H^+$ antiport activity of SOS1 in vitro (Qiu et al., 2002), suggesting that SOS2 directly regulates the activity of SOS1. To determine if in vivo SOS2 kinase activity is sufficient to regulate SOS1 activity, and if SOS1 activation might contribute to the improved salt tolerance conferred by T/DSOS2, we measured SOS1 transport activity in the *35S::T/DSOS2* transgenic plants and the untransformed wild-type, *sos2-2* and *sos3-1* control plants. For these studies, highly purified plasma membrane vesicles were isolated from wild-type, *sos2-2*, *sos3-1*, and their T/DSOS2 transgenic plants after treatment with 250 mM NaCl for 3 days. When T/DSOS2 protein was added in vitro to plasma membrane vesicles isolated from untransformed wild-type plants, $Na^+/H^+$-exchange activity increased with increasing NaCl concentration and was higher than activity in the absence of T/DSOS2 protein at all NaCl concentrations (Figure 9A). A maximum stimulation of activity of 40% relative to activity without added protein was measured with 100 mM NaCl. $Na^+/H^+$ exchange activity, measured in vesicles isolated from T/DSOS2 transgenics of wild-type, *sos2* and *sos3* plants, was higher than in the respective untransformed controls (Figure 9B to 9D); however, the exchange activity of the *sos2-2*, and *sos3-1* transgenic lines was restored to only half to two-thirds of the levels of activity measured in untransformed wild type, in agreement with the partial suppression of their salt sensitivity (Figure 5). These results demonstrate that

expression of the active kinase T/DSOS2 enhanced SOS1 activity in vivo in the transgenic plants. These measurements also provide further evidence that more than SOS2 is required for full SOS1 activity and salt tolerance in vivo.

**The C-terminal region of SOS2 is required for function in planta**

The above experiments showed that the active kinase T/DSOS2 could enhance SOS1 activity and salt tolerance when expressed either in wild-type, *sos2* or *sos3* plants. Since T/DSOS2/308 (with the $Thr^{168}$ to Asp change and in which the FISL domain and C-terminal 117 amino acids were removed) exhibited the highest protein kinase activity in vitro (Figure 1) and was the most active in activating SOS1 function in yeast (Figure 2), *T/DSOS2/308* was expressed in the *sos2-2* or *sos3-1* mutants under the CaMV 35S promoter. Twenty-four independent $T_2$ transgenic lines from each transformation were tested for growth in salt; and none had salt tolerance that was greater than that of either the *sos2-2* or *sos3-1* mutant. One representative $T_3$ homozygous line from expression of T/DSOS2/308 in *sos2-2* (Figure 10A and 10B, upper) and *sos3-1* (Figure 10A and 10B, lower) is shown. Although the transgene was expressed at high levels in the transgenic plants (Figure 10C), salt tolerance was not enhanced. These results suggest that the FISL motif and/or the C-terminal 117 amino acids are required for salt tolerance in planta.

Compared to T/DSOS2/308, T/DSOS2/329 (with the $Thr^{168}$ to Asp change and in which the C-terminal 117 amino acids were removed) contains the FISL motif but is not as active because the FISL motif is inhibitory to SOS2 activity (Figure 1). When *35S::T/DSOS2/329* was expressed in the *sos2-2* or *sos3-1* mutants, the salt-tolerance was not restored. One representative $T_3$ homozygous line from expression of T/DSOS2/329 in *sos2-2* (Figure 10D and 10E, upper) and *sos3-1* (Figure 10D and 10E, lower) is also shown. As with T/DSOS2/308,

expression of the transgenes was high in the transgenic plants (Figure 10F) but salt tolerance was not enhanced. The data from the analysis of the *sos2-2* and *sos3-1* transgenic lines expressing T/DSOS2/329 suggest that adding back the FISL motif is not sufficient to restore the function to the active T/DSOS2/308 kinase in planta. Together with the data from the T/DSOS2/308 expressing transgenic plants, the results reveal a critical role for the C-terminal region of SOS2 in salt tolerance in planta.

To further examine the role of the FISL motif and the C-terminal 117 residues, T/DSOS2DF (with the $Thr^{168}$ to Asp change and in which the FISL domain was removed) was expressed in wild-type Arabidopsis and the *sos2-2* or *sos3*-1 backgrounds. When T/DSOS2DF was expressed in wild-type plants, four of twelve of $T_2$ transgenic lines evaluated were more salt tolerant than the untransformed wild type. The levels of *T/DSOS2DF* transcript were determined in two $T_3$ homozygous lines and high accumulation in both was detected (Figure 11A). When these plants were evaluated for salt tolerance during germination and seedling growth, no significant differences in germination were detected on medium without salt (Figure 11B), in contrast, the transgenic plants had faster germination and improved seedling development on MS medium containing 100 mM NaCl (Figure 11C). When five-day-old seedlings were transferred to MS medium, the growth of wild-type and transgenic plants was similar (Figure 11D). When seedlings were transferred to MS medium containing 100 mM or 120 mM NaCl, growth of the transgenic plants was less inhibited by NaCl (Figure 11E and 11F).

We attempted to enrich the T/DSOS2DF protein by incubating total protein extracts (from a transgenic line with increased salt tolerance) with GST-SOS3 on glutathione-Sepharose beads. However, T/DSOS2DF protein could not be detected by immunoblot analysis (data not shown), and no T/DSOS2DF kinase activity was detected in peptide phosphorylation assays

(data not shown), indicating that T/DSOS2DF did not interact with SOS3 and further supporting previous interaction studies suggesting that the FISL motif is required for SOS2/SOS3 interaction.

When T/DSOS2DF was expressed in the *sos2-2* and *sos3-1* backgrounds, three of twelve $T_2$ *sos2-2* transgenic lines had salt tolerance that was restored almost to wild-type levels. However, of the twenty-four $T_2$ *sos3-1* transgenic lines evaluated, all had the *sos3-1* phenotype with only slight root bending. Representative $T_3$ homozygous *sos2-2* and *sos3-1* transgenic lines are shown in Figure 12. The *T/DSOS2DF* transcript was detected in both transgenic lines (Figure 12F). When five-day-old seedlings from wild-type, *sos2-2*, *sos3-1* and transgenic *sos2-2* or *sos3-1* lines were transferred to MS medium without salt, no significant differences in growth were found (Figure 12A and 12C). However, when the seedlings were transferred to MS medium containing 100 mM NaCl, *sos2-2* plants died within two weeks while the phenotype of the *sos2-2* transgenic plants was similar to wild type but with slightly smaller shoots and fewer lateral roots (Figure 12B). Expression of T/DSOS2DF in *sos3-1* led to a slight increase in root elongation relative to *sos3-1* when plants were grown on 100 mM NaCl (Figure 12D and 12E); however both *sos3-1* and the *sos3-1* transgenic lines were unable to survive on this medium for more than two weeks.

The results with the *35S::T/DSOS2DF* transgenic plants demonstrate that the 117 residues C-terminal to the FISL motif are necessary and sufficient for the in planta function of the active SOS2 kinase proteins in wild-type and *sos2-2* mutant plants. However, function of the active kinase in *sos3-1* mutant plants appears to require the FISL motif as well. Improved salt tolerance in the wild-type transgenic plants provides further support that the kinase activity of SOS2 is limiting in vivo and increasing this activity can be beneficial for salt tolerance.

## DISCUSSION

Genetic analysis of *sos1*, *sos2*, and *sos3* mutants suggested that SOS1, SOS2, and SOS3 function in the same pathway for $Na^+$ homeostasis in Arabidopsis (Zhu et al., 1998). SOS2 is activated by its interacting-protein SOS3 in a calcium-dependent manner (Halfter et al., 2000). When expressed in yeast, the SOS3-SOS2 complex phosphorylates and activates SOS1 to enhance $Na^+$ efflux and NaCl tolerance (Quintero et al., 2002). The $Na^+/H^+$ exchange activity of SOS1 is substantially diminished in *sos2* and *sos3* mutant plants, and in vitro addition of the activated form of SOS2, T/DSOS2DF, rescues the exchange activity in not only *sos2* but also *sos3* plasma membrane vesicles (Qiu et al., 2002). Therefore, the requirement of SOS3 in vitro for SOS1 activation can be bypassed by the activated SOS2 protein. Results presented here demonstrate that in yeast, the requirement of SOS3 in salt tolerance can also be partially bypassed in vivo by the activated forms of SOS2. However in planta, only the activated form of SOS2 that retains structural integrity (i.e. T/DSOS2) can bypass the requirement for SOS3. These results show that data obtained in vitro and even in vivo from a heterologous system only partially reflect what happens in planta. The in planta experiments thus reveal new functions of the regulatory proteins and their essential structural domains.

The activity and functionality of the different forms of SOS2 in vitro, in yeast and in wild-type and mutant Arabidopsis is summarized in Table 1. In yeast, the effect of the kinase forms on SOS1 activation and salt tolerance is largely correlated with their in vitro kinase activities. For example, the T/DSOS2/308 form is most active in vitro, and is also most effective in enhancing the salt tolerance of the yeast cells. In contrast, when expressed in Arabidopsis,

only the T/DSOS2 form was able to partially rescue the *sos3-1* mutant phenotype, thus bypassing the requirement of SOS3 in planta. Although T/DSOS2/308, T/DSOS2/DF and T/DSOS2/329 are all more active in vitro, they are not able to bypass the SOS3 deficiency in planta. The *sos3-1* mutation causes a deletion of three amino acids in one of the EF-hands of SOS3 that reduces but does not eliminate the calcium binding of SOS3 (Liu and Zhu, 1998; Ishitani et al., 2000). It is therefore possible that this mutant form of SOS3 is still partially functional. Since T/DSOS2DF, which has only the FISL motif removed from T/DSOS2, does not suppress the *sos3-1* mutation whereas T/DSOS2 does, it is possible that the mutant polypeptide SOS3-1 still binds to T/DSOS2 and targets the activated kinase to the plasma membrane and the phosphorylation of SOS1. However, the mutant SOS3-1 protein has been previously shown not to interact with SOS2 in a yeast two-hybrid assay (Ishitani et al., 2000). Alternatively, T/DSOS2 could interact with another SCaBP and be targeted to the plasma membrane in the absence of SOS3. However, this later speculation is also inconsistent with the finding that T/DSOS2DF partially rescues the *sos2-2* mutant phenotype since deletion of the FISL motif abrogates interaction with SOS3 and other ScaBPs. A better knowledge of the various functional domains of SOS2 and SOS3 and related proteins will be needed to fully understand the complexity of this pathway. Nevertheless, the observations collectively reveal a requirement of the C-terminal regulatory region of SOS2 for salt tolerance in planta.

Another unexpected observation is that T/DSOS2 partially rescues the salt hypersensitivity in the shoot but not the root in *sos2* and *sos3* mutants. The lack of effect in the root is not likely explained by the use of the CaMV 35S promoter, since wild-type SOS2 expressed under the same promoter does rescue the *sos2* mutant in both the shoot and root. A root-specific regulation of SOS2 may occur through its activation loop, and the T/D mutation

may interfere with such a regulation. Although the hypothetical upstream protein kinase(s) for SOS2 has not been identified, it is conceivable that there might be a root-specific isoform of such a kinase. On the other hand, expression of T/DSOS2DF can rescue the *sos2* mutant phenotype. Thus, if the hypothetical root-specific upstream kinase is responsible for the inactivity of T/DSOS2 in the root, it must not have an effect on T/DSOS2DF.

Regulatory genes are often considered superior targets of biotechnological applications for plant improvements, because they control many downstream effector genes. For example, ectopic expression of the CBF/DREB1A family of transcription factors and the MAPKKK ANP1 have been shown to substantially improve plant tolerance to various abiotic stresses (Jaglo-Ottosen et al., 1998; Gilmour et al., 2000; Kovtun et al., 2000). SOS2 is regulator of ion transporters (Zhu, 2002), some of which have been shown to confer increased salt tolerance when over-expressed in transgenic plants (Apse et al., 1999; Shi et al., 2003). In this study, we evaluated the feasibility of using SOS2 to improve plant salt tolerance. Over-expression of wild-type SOS2 did not confer any increased salt tolerance in transgenic Arabidopsis. However, ectopic expression of the activated forms T/DSOS2 and T/DSOS2/DF, led to measurable enhancement in salt tolerance in transgenic Arabidopsis. These results raise the hope that by exploring various versions of the protein kinase, an effective allele may be created that might become useful even in field conditions. The lack of any beneficial effect of the T/DSOS2/308 form in wild-type Arabidopsis also reinforces the notion that the C-terminal region of SOS2 is critical for function in planta.

## MATERIALS AND METHODS

**Preparation of active SOS2 kinase expression plasmids and plant transformation**

For expression of constitutively active SOS2 kinase in Arabidopsis, DNA fragments of *T/D SOS2*, *T/DSOS2/308*, *T/DSOS2/329*, and *T/DSOS2DF* were digested from their GST-fusion constructs (Guo et al., 2001) with *Bam*HI and *Eco*RI, and cloned into a binary vector (pCAMBIA1027) under the control of the CaMV 35S promoter. The plasmids were introduced into *Agrobacterium* strain GV3101 by electroporation and then transferred into wild-type (Columbia ecotype), *sos2-2*, or *sos3-1* mutant plants by vacuum-infiltration. Hygromycin-resistant transgenic $T_2$ and $T_3$ plants were tested for growth in salt.

**Growth measurements**

Seeds of wild-type, *sos2-2*, *sos3-1* and transgenic plants were surface-sterilized in 7% (w/v) hypochlorite and 0.01% (w/v) Triton X-100 and then rinsed five times with sterile water. The seeds were sown on a Murashige-Skoog (MS) nutrient medium containing 0.6% agar and the indicated NaCl concentrations. The seeds were stratified at 4°C for 3 days and then transferred to 22°C under continuous light for measurements of germination and growth.

For seedling growth in salt, 5-day-old seedlings of wild-type, *sos2-2*, *sos3-1* and transgenic plants were transferred to MS medium containing 1.2% agar and the indicated NaCl concentrations. Growth was monitored using a root bending assay (Zhu et al., 1998). Plant salt tolerance in soil was assayed as described in Shi et al. (2003).

**RNA analysis**

Total RNA was extracted from two-week-old seedlings and 40 μg of each sample was used for RNA analysis as described (Guo et al., 2001).

**Immunoblot analysis and kinase assays**

Total proteins (five grams from ten-day-old seedlings) were extracted at 4°C from wild-type, *sos2-2*, *sos3-1* and transgenic plants in 10 ml 1× PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.4 mM $NaH_2PO_4$, pH7.4) with 5 mM dithiothreitol, 2 μg aprotinin $mL^{-1}$, 2 μg leupeptin $mL^{-1}$, and 2 mM phenylmethanesulfonyl fluoride. To isolate sufficient amounts of (T/D)SOS2 protein, GST-SOS3 fusion protein (Halfter et al., 2000) was first purified using glutathione-Sepharose beads (Amersham Pharmacia). Total Arabidopsis proteins were then incubated with 100 μl of GST-SOS3 coupled to the Sepharose beads for two hours at 4°C. The GST-SOS3 beads-(T/D)SOS2 protein complex was washed three times with 1×PBS buffer. Ten μl of the protein complex were used for either immunoblot analysis or protein kinase assays.

For immunoblot analysis, 3 μl of 3× protein loading buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 30% glycerol, 1.5% β-mercaptoethanol, and 0.3% bromophenol blue) were added to 10 μl protein and the samples were boiled for 5 min. The samples were run on a 10% SDS-PAGE gel and the proteins were transferred to a pure nitrocellulose membrane (Bio-Rad Laboratories) at 80 volts for 60 min. The membrane was blocked overnight at 4°C in 1×PBS buffer with 5% fat free milk, rinsed one time with 1× PBS and incubated with SOS2 antibodies (diluted 1:1000) for 3 hr at room temperature. After three washes with 1×PBS buffer, the membrane was incubated with anti-rabbit IgG secondary antibody diluted 1:2500 (Amersham, Life Science) for 1 hour at room temperature. The membrane was then washed five times with 1×PBS and the

immunoreactive bands detected using the chemiluminescent ECL detection substrate (Amersham Pharmacia Biotech).

Ten µl of SOS3-(T/D)SOS2 beads were used for p3 peptide phosphorylation assays as described in Halfter et al. (2000).

**$Na^+/H^+$ antiport**

Plasma membrane vesicles were isolated using aqueous two-phase partitioning as described (Qiu and Su, 1998; Qiu et al., 2002). $Na^+/H^+$ antiport activity was measured as a $Na^+$-induced dissipation of the pH gradient (ΔpH, i.e., a $Na^+$-induced increase in quinacrine fluorescence; Qiu et al., 2002). When ΔpH reached steady state, NaCl was added to initiate $Na^+$ transport. To determine initial rates of $Na^+/H^+$ exchange (change in fluorescence per minute; $\Delta\%F\ min^{-1}$), changes in relative fluorescence were measured during the first 15 s after addition of $Na^+$. Specific activity was calculated by dividing the initial rate by the mass of plasma membrane protein in the reaction ($\Delta\%F\ mg^{-1}$ protein $min^{-1}$). To determine whether T/DSOS2 activates SOS1 in vitro, 200 ng of T/DSOS2 protein was pre-incubated with wild-type membrane vesicles for 7 min at room temperature before the antiport activity assays.

**Yeast growth**

The yeast expression plasmid, pSOS1, was constructed previously (Quintero et al. 2002). The plasmids that contain either wild-type or active forms of *SOS2* (*T/DSOS2*, *T/DSOS2/308*, and *T/DSOS2DF*) were made by inserting *Bam*HI-*Eco*RI fragments from pGEX-SOS2 derivatives (Guo et al. 2001) into the *Bam*HI-*Eco*RI site of the p424 GPD vector. Transformation of yeast was carried out with yeast strain AXT3K (Quintero et al. 2002) using a standard PEG method.

The ability of yeast to grow in salt was tested on synthetic complete (SC) medium lacking uracil, tryptophan, histidine, and leucine and supplemented with the indicated concentrations of NaCl. The $K^+$ concentration in SC medium was 18 mM. Strains were cultured overnight in AP (Quintero et al., 2002) liquid medium with 1 mM KCl. After harvest, cells were resuspended and diluted decimally in distilled water. Aliquots (5 μl) were spotted onto SC with agar and grown for 4 days at 30°C.

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**Table 1. Summary of the in vitro and in vivo activities of wild-type and activated forms of SOS2**

The level of activity is indicated by the number of + signs. nd, not determined.

| | Auto-phosphorylation | Peptide phosphorylation | Yeast growth (in salt) | Arabidopsis growth (in salt) | | |
|---|---|---|---|---|---|---|
| | | | | *sos2* | *sos3* | WT |
| SOS2 | - | - | + | + | - | - |
| T/DSOS2 | ++ | ++ | + | + | + | + |
| T/DSOS2DF | ++ | +++ | +++ | + | - | + |
| T/DSOS2/329 | + | + | nd | - | - | nd |
| T/DSOS2/308 | +++ | ++++ | ++++ | - | - | nd |

## FIGURE LEGENDS

**Figure 1. Active SOS2 kinases. (A)** Model of the domains of wild-type and altered SOS2 protein kinases. The kinase activities (autophosphorylation and phosphorylation of an in vitro substrate) of altered forms of SOS2 (GST-fusion proteins of T/DSOS2, T/DSOS2/308, T/DSOS2DF, T/DSOS2/329) were evaluated. After the autophosphorylation assays, protein was separated by SDS/PAGE and the gel was stained with Coomassie blue **(B)** and exposed to x-ray film **(C)**. The ability of the same GST-SOS2 fusion proteins to phosphorylate the peptide substrate p3 (400 pmol per assay) was determined **(D)**.

**Figure 2. Active SOS2 kinases increase the NaCl tolerance of yeast expressing SOS1.** Wild-type SOS2 or active SOS2 kinases were co-transformed with SOS1 into yeast AXT3K cells and grown overnight in liquid AP medium with 1 mM KCl. Five microliters of serial decimal dilutions of the overnight cultures were spotted onto plates containing SC alone or supplemented with 150, 300, 450 or 600 mM NaCl. Plates were incubated at 28°C and photographed after 4 days.

**Figure 3. Expression of SOS2 complements the *sos2-2* salt-sensitive phenotype, but not the *sos3-1* salt-sensitive phenotype.** Five-day-old seedlings grown on MS agar medium were transferred to MS agar medium without NaCl **(A)** and **(C)** or with 100 mM NaCl **(B)** and **(D)**; photographs were taken 10 days after transfer. **(E)** SOS2 transcript levels in *sos2-2*, *sos3-1*, and *35S::SOS2* transgenic lines. RNA blot analysis with total RNA extracted from (1) *sos2-2*, (2)

*sos3-1* or (3) *sos2-2* and (4) *sos3-1* transgenic plants grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control. WT, wild type.

**Figure 4. Expression of T/DSOS2 in *sos2-2* and *sos3-1*.** **(A)** RNA blot analysis of *T/DSOS2* expression in *sos3-1*, *sos2-2* or *sos3-1* and *sos2-2* transgenic lines grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control. Total protein was extracted from mutant and transgenic plants and incubated with GST-SOS3 coupled to glutathione-Sepharose beads. The GST-SOS3-T/DSOS2/SOS2 complex was used for **(B)** immunoblot analysis with: protein from (1) *sos3-1* and (3) *sos2-2* mutants, or (2) *sos3-1* and (4) *sos2-2* transgenic lines. Proteins were probed with anti-SOS2 antibody. The GST-SOS3-T/DSOS2/SOS2 complex was also used for **(C)** peptide phosphorylation assays with protein from (1) *sos3-1*, (2) the *sos3-1* transgenic line, (3) *sos2-2* and (4) the *sos2-2* transgenic line.

**Figure 5. Expression of T/DSOS2 partially rescues the *sos2-2* and *sos3-1* salt-hypersensitive phenotypes.** Five-day-old seedlings grown on MS agar medium were transferred to MS agar medium without NaCl **(A)** or with 100 mM NaCl **(B)**; photographs were taken 10 days after transfer. **(C)** Fresh weight (in mg) of 5 plants of (1) wild type, (2) *sos3-1*, (3) a *sos3-1* transgenic line, (4) *sos2-2*, and (5) *sos2-2* transgenic line two weeks after transfer to MS + 100 mM NaCl (means ± standard error of three replicate experiments). Growth of **(D)** *sos2-2* and a *sos2-2* transgenic line, and **(E)** *sos3-1* and a *sos3-1* transgenic line in soil in which the NaCl levels were increased by 50 mM every 4 days until a final concentration of 200 mM was reached. Photographs were taken after 15 days in 200 mM NaCl.

**Figure 6. Expression of SOS2 does not increase salt tolerance in Arabidopsis. (A)** *SOS2* transcript levels wild type and three *35S::SOS2* transgenic lines grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control. Seeds from wild type (top) and two *35S::SOS2* transgenic lines (bottom) were germinated on MS medium **(B)** or MS + 100 mM NaCl **(C)**; photographs were taken 5 days (left panel) and 10 days (right panel) after germination. Five-day-old seedlings grown on MS agar medium were transferred to MS agar without NaCl **(D)**, or with 50 **(E)** or 100 mM NaCl **(F)**; photographs were taken 10 days after transfer.

**Figure 7. Expression of T/DSOS2 in Arabidopsis. (A)** *T/DSOS2* transcript levels in wild type and WT/T/DSOS2 transgenic lines. RNA blot analysis with total RNA extracted from wild type and two WT/T/DSOS2 lines grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control. Total protein was extracted from wild type and transgenic plants, and incubated with GST-SOS3 coupled to glutathione-Sepharose beads. The GST-SOS3-T/DSOS2/SOS2 protein complex was used for immunoblot analysis with anti-SOS2 antibody **(B)** and peptide phosphorylation assays **(C)**.

**Figure 8. Expression of T/DSOS2 increases salt tolerance in Arabidopsis. (A)** Seeds from wild type (top) and two WT/T/DSOS2 transgenic lines (bottom) were germinated on MS medium (left panel) or MS + 100 mM NaCl (right panel); photographs were taken 5 days (left panel) and 10 days (right panel) after germination. **(B)** Five-day-old seedlings from wild type or WT/T/DSOS2 transgenic lines grown on MS agar were transferred to MS agar without NaCl (top), with 100 (middle), or 120 mM (bottom) NaCl; photographs were taken 15 days after

transfer. **(C)** Wild-type and transgenic plants were grown in soil in which the NaCl levels were increased by 50 mM every 4 days until a final concentration of 200 mM was reached. Photographs were taken after 15 days in 200 mM NaCl.

**Figure 9. Active T/DSOS2 increases plasma membrane $Na^+/H^+$-exchange activity in vitro and in vivo. (A)** When added in vitro, T/DSOS2 protein stimulates plasma membrane $Na^+/H^+$-exchange activity in vesicles isolated from wild-type plants. Transport assays were performed as described in Materials and Methods. The pH gradient (ΔpH) was formed in the absence (•) or presence (○) of T/DSOS2 protein. When ΔpH reached steady state, NaCl was added over a range of final concentrations (0-100 mM), and the initial rates of dissipation ($Na^+/H^+$ exchange) were measured. When compared to activity in wild type, *sos2* and *sos3*, plasma membrane $Na^+/H^+$-exchange activity is higher in wild type **(B)**, *sos2* **(C)** and *sos3* **(D)** plants over-expressing T/DSOS2. Assays were performed using vesicles isolated from wild type (•) and transgenic (○) plants. When ΔpH reached steady state, NaCl was added over a range of final concentrations (0-100 mM), and the initial rates of dissipation were measured. Units of $Na^+/H^+$ exchange are $\Delta\%F\ mg^{-1}$ protein $min^{-1}$. Data in panels A-D represent means ± standard error of at least three replicate experiments. Each replicate experiment was performed using independent membrane preparations.

**Figure 10. Expression of T/DSOS2/308 or T/DSOS2/329 does not complement the *sos2-2* and *sos3-1* salt-sensitive phenotypes.** Five-day-old seedlings grown on MS agar medium were transferred to MS agar medium without NaCl **(A)** and **(D)** or with 100 mM NaCl **(B)** and **(E)**; photographs were taken 10 days after transfer. *T/DSOS2/308* **(C)** or *T/DSOS2/329* **(F)** transcript

levels in (1) *sos2-2*, (2) *sos3-1* or (3) *sos2-2* and (4) *sos3-1* transgenic lines grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control.

**Figure 11. Expression of T/DSOS2DF increases salt tolerance in Arabidopsis. (A)** *T/DSOS2DF* transcript levels in wild type and two transgenic lines grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control. Seeds from wild type (top) and two transgenic lines (bottom) were germinated on MS medium **(B)** or MS + 100 mM NaCl **(C)**; photographs were taken 5 days (left panel) and 10 days (right panel) after germination. Five-day-old seedlings grown on MS agar medium were transferred to MS agar without NaCl **(D)**, or with 100 **(E)** or 120 mM NaCl **(F)**; photographs were taken 10 days (D) and 15 days (E and F) after transfer.

**Figure 12. Expression of T/DSOS2DF rescues the *sos2-2* salt-hypersensitive phenotype, but not the *sos3-1* salt-hypersensitive phenotype.** Five-day-old seedlings grown on MS agar medium were transferred to MS agar medium without NaCl **(A)** and **(C)** or with 100 mM NaCl **(B)** and **(D)**; photographs were taken 10 days after transfer. **(E)** Root growth (in cm) of (1) wild type, (2) *sos3-1*, (3) a *sos3-1* transgenic line, (4) *sos2-2*, and (5) a *sos2-2* transgenic line grown on MS + 100 mM NaCl for two weeks (means ± standard error of three replicate experiments). **(F)** *T/DSOS2DF* transcript levels. RNA blot analysis with total RNA extracted from: (1) *sos2-2*, (2) *sos3-1*, (3) a *sos2-2* transgenic and (4) a sos3-1 transgenic line grown in the absence of NaCl. 25S rRNA (ethidium bromide stained) was used as a loading control.